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Molecular regulation by H_2S of antioxidant and glucose metabolism in cold-sensitive *Capsicum*

Xue Ping Song[1†], Bi Yan Cao[1†], Ze Ping Xu[1†], Le Liang[1], JiaChang Xiao[1], Wen Tang[1], Ming Hui Xie[1], Dong Wang[1], Li Zhu[2], Zhi Huang[1], Yun Song Lai[1], Bo Sun[1], Yi Tang[1] and Huan Xiu Li[1*]

Abstract

Background Cold is an important environmental limiting factor affecting plant yield and quality. *Capsicum* (chili pepper), a tropical and subtropical vegetable crop, is extremely sensitive to cold. Although H_2S is an important signaling regulator in the responses of plant growth and development to abiotic stress, few studies have examined its effects on cold-sensitive capsicum varieties. Through biotechnology methods to enhance the cold resistance of peppers, to provide some reference for pepper breeding, investigated molecular regulation by H_2S of responses to cold stress in cold-sensitive capsicum plants, via physiological and transcriptomic analyses.

Results In capsicum seedlings, exogenous H_2S enhanced relative electrical conductivity (REC) and levels of malondialdehyde (MDA) under cold stress, maintained membrane integrity, increased the activity of enzymatic and non-enzymatic antioxidants, balanced reactive oxygen species levels ($O_2^{\cdot-}$ and H_2O_2), and improved photosynthesis, mitigating the damage caused by cold. In addition, 416 differentially expressed genes (DEGs) were involved in the response to cold stress after H_2S treatment. These DEGs were mainly enriched in the ascorbate–glutathione and starch–sucrose metabolic pathways and plant hormone signal-transduction pathways. Exogenous H_2S altered the expression of key enzyme-encoding genes such as *GST*, *APX*, and *MDHAR* in the ascorbate–glutathione metabolism pathway, as well as that of regulatory genes for stimulatory hormones (auxin, cytokinins, and gibberellins) and inhibitory hormones (including jasmonate and salicylic acid) in the plant hormone signal-transduction pathway, helping to maintain the energy supply and intracellular metabolic stability under cold stress.

Conclusions These findings reveal that exogenous H_2S improves cold tolerance in cold-sensitive capsicum plants, elucidating the molecular mechanisms underlying its responses to cold stress. This study provides a theoretical basis for exploring and improving cold tolerance in capsicum plants.

Keywords Cold stress, H_2S, Enzymatic antioxidant, Non-enzymatic antioxidant, Photosynthesis, Transcriptome

†Xue Ping Song, Bi Yan Cao and Ze Ping Xu, equally contributed to this work and are co-first authors.

*Correspondence:
Huan Xiu Li
huanxiuli62@163.com

[1]College of Horticulture, Sichuan Agricultural University, Chengdu, Sichuan 611130, China
[2]Sichuan Academy of Agricultural Characteristic Plants, No.14 Yongxing Road, Chonglong Town, Zizhong County, Neijiang City, Sichuan Province 641200, China

Introduction

Temperature is a major environmental factor affecting plant growth and development. Excessively high or low environmental temperatures can affect plants. Extensive research has shown that cold stress significantly affects the quality and yield of grain, oil, and horticultural crops [1]. the vegetable *Capsicum annuum* L. (capsicum) contains multiple vitamins, with vitamin C being the most abundant. It can be used fresh or as a spice during the green and ripe stages. Dried capsicum and capsicum powder are rich in vitamins and capsaicin, making them important export products in China [2]. capsicum is are highly sensitive to temperature; seedlings exposed to temperatures below 5–10 °C can suffer from cold damage, preventing them from safely overwintering or withstanding frost in early spring [3]. Plants subjected to cold stress rapidly accumulate large amounts of reactive oxygen species (ROS), primarily the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals, and singlet oxygen, which are highly oxidative. Excessive ROS levels can disrupt the dynamic balance of the cellular environment and impair metabolic functions [4, 5]. When plants are subjected to abiotic stress, their enzymatic and non-enzymatic systems are rapidly activated. NADPH oxidase catalyzes electron transfer from the cytoplasm to O_2, leading to the formation of $O_2^{\cdot-}$ [6]. Dismutation then occurs spontaneously or via the action of SOD [7], generating H_2O_2. APX then converts H_2O_2 to H_2O with high affinity, thereby alleviating excessive ROS levels and reducing damage to the plants [8]. Studies have found that cold stress can damage the photosynthetic organs of plants, affecting the opening and closing of leaf stomata, impairing CO_2 absorption, damaging the thylakoid membranes of chloroplasts, affecting the reaction centers of photosystems, and inhibiting the activity of key enzymes in the Calvin cycle [9]. Photosynthesis-related metabolism typically involves the synthesis of starch, which helps maintain respiration in the dark, enabling symbionts to supply sugars during both the day and night [10]. Sucrose, the primary organic carbon source, is transported from photosynthetic leaves to non-photosynthetic tissue via the phloem. In the phloem, sucrose is hydrolyzed into glucose and fructose by invertase (*INV*) after phloem unloading, or is degraded into UDP glucose and fructose by sucrose synthase (*SUS*). Sucrose can be degraded into hexoses for various purposes [11]. In addition to supporting nighttime metabolic functions, starch rapidly degrades under light conditions, aiding the production of organic acids and sugars, increasing the turgor pressure of guard cells and promoting stomatal opening [12]. The key starch-degrading enzyme involved in this process is β-amylase 1 (*BAM1*), which works in conjunction with α-amylase 3 (*AMY3*) to degrade starch. *Arabidopsis* mutants lacking *AMY3* show a substantial reduction in osmotic-stress-induced starch degradation, resulting in reduced accumulation of sugars and proline (Pro) and reduced water-uptake capacity [13, 14].

H_2S, a signaling molecule in plants, participates in various activities such as flowering, fruit setting, senescence, photosynthesis, and sucrose and starch synthesis and degradation [15, 16]. Exogenous H_2S can increase endogenous H_2S levels and the activity of key enzymes, and alters the responses of multiple signaling pathways to abiotic stress. For example, following salt stress, H_2S works synergistically with Ca^{2+} to increase the concentration of sodium and potassium ions on both sides of the membrane, maintaining membrane lipid integrity and redox homeostasis [17]. Exogenous H_2S can enhance the resistance of grapes to stress by inducing superoxide dismutase (SOD) activity and *ICE1* and *CBF3* expression [18]. Spraying bananas with H_2S during storage can increase their osmotic substance levels and oxidase activity, helping to maintain their quality and extending their shelf life by enhancing stress resistance and delaying ripening [19]. H_2S participates in regulating the upstream and downstream signaling of various plant hormones. In cucumber explants, IAA (indole-3-acetic acid) depletion inhibits adventitious root formation, whereas administration of H_2S alleviates this inhibition [20]. In maize seedlings, H_2S acts as a downstream signaling molecule in salicylic acid (SA)-induced heat tolerance. In *Arabidopsis*, H_2S induces stomatal movement via an abscisic acid (ABA)-acid-dependent pathway [21].

In recent years, with the increasing severity of cold stress, capsicums, whether used as vegetables or spices, hold a significant market share. However, due to their preference for warm conditions, they cannot safely overwinter during the winter months. To ensure a steady supply of peppers during winter, developing cold-tolerant varieties has become one of the primary goals for breeders. However, research on H_2S in cold-sensitive chili capsicum seedlings is relatively limited. This study investigates the effects of H_2S pretreatment followed by short-term cold stress on chili capsicum seedlings, focusing on major physiological, biochemical, and transcriptomic changes. It analyzes the molecular mechanisms of differential gene expression, enzymatic systems, and starch-sucrose metabolism pathways in response to H_2S after low-temperature exposure, providing a reference for future research.

Materials and methods

Plant materials

The experiment was carried out in the vegetable Laboratory of Horticulture College of Sichuan Agricultural University in January 2022. Under cold stress, an artificial climate box (Model: RGX300EF, Tianjin Test Instrument Co., LTD., Tianjin, China.) was used to set the

temperature at 5 ℃ for 48 h. The experimental material was a cold-sensitive pepper (Dongxing capsicum) screened in our laboratory, and the seeds were stored at the Vegetable Laboratory of Horticulture College, Sichuan Agricultural University. NaHS, an H_2S donor, was purchased from McLean. Hypohtrine (HT), an H_2S scavenger, was purchased from Sigma Aldrich (St Louis, MO, USA).

Experimental design

Full and intact capsicum seeds were selected and sown in a 12×6 well seedling tray in growth medium (nutrient soil: vermiculite, 5:5 v/v). Seedlings were grown at 25/18°C (day/night) with a 12 h /12 h light / dark photoperiod (light intensity, 300 μmol m^{-2} s^{-1}; relative humidity, 75%). Once the seedlings had 3– 4 true leaves, they were transplanted into 10×10×15 cm pots. Once they had 7–8 true leaves, the exogenous treatment was applied continuously for 3 d to prepare them for cold stress. These temperature, humidity, and light conditions were maintained throughout the seedling stage. The cold stress temperature was set to 5 ℃ (±1 °C).

The experimental design involved four treatments: Normal temperature control (with 200 mL water), at 25 °C ('Control'); three cold treatments were used, namely cold stress alone (with 200 mL water; 'Cold'), or cold stress with 200 mL of NaHS pretreatment at 0.5 mmol·L^{-1} ('H_2S') or 200 mL of HT pretreatment at 0.2 mmol·L^{-1} ('HT'). Each treatment was 15 plants and repeated 3 times. The exogenous substances were applied continuously for 3 d. The following day, the seedlings were placed in a growth chamber to induce cold stress. Leaf samples were collected for physiological and transcriptomic analyses at different time points (0, 6, 12, 24, and 48 h). Each sample was bioreplicated three times.

Determination of physiological and biochemical indices

Fresh leaves (0.1 g) were placed in a test tube containing 30 ml of deionized water and allowed to stand for 6 h. The conductivity of deionized water (R_0) was measured using a conductivity meter. The conductivity of the sample solution was measured before and after boiling, recorded as R_1 and R_2, respectively. The relative electrolyte leakage (REC) was calculated using the formula: REC (%) = (R_1 – R_0) ×100/ (R_2 - R_0). Malondialdehyde (MDA) content was determined using the thiobarbituric acid method, and proline (Pro) content was measured using the sulfosalicylic acid method [22]. Superoxide anion (O_2·$^-$) content was assessed using the p-aminobenzoic acid method [23], and hydrogen peroxide (H_2O_2) content was measured using the potassium iodide spectrophotometric method [24]. The activities of peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) were determined using guaiacol, nitroblue tetrazolium (NBT), and UV absorbance methods, respectively [25]. The activities of Monodehydroascorbate reductase (MDAHR) at 340 nm and Dehydroascorbate reductase (DHAR) at 265 nm were also measured [26]. Ascorbic acid (AsA), dehydroascorbic acid (DHA), glutathione (GSH), and glutathione disulfide (GSSG) contents were determined following the methods described by Noctor and Foyer [27].

The photosynthetic parameters were determined using a Li-Cor 6400XT (Gene Company Limited, Hong Kong, China) portable photosynthesis measurement system. Photosynthetic indices were measured using fully unfolded leaves. With a red/blue LED as the light source, the flow rate was 500 mL min^{-1}, the CO_2 concentration was 400 μmol^{-1}, and the PAR was 1000 μmol m^{-2} s^{-1}. The photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (Tr), and intercellular CO_2 concentration (Ci) of the best functional leaves (3rd to 4th true leaves) of pepper seedlings in each treatment were determined, and each treatment was repeated three times. The enzyme activity changes of RubisCO, FBA, and SBP were determined using an ELISA kit (Shanghai Fwei Biotechnology Co., Ltd., Fengxian District, Shanghai, China).

The determination of endogenous H_2S followed Sekiya et al.'s methylene blue method [28]. 0.1 g fresh chilli leaves were ground into homogenization on ice with 0.9 ml 20 mM pre-cooled Tris-HCl buffer (pH 8.0), and then centrifuged at 4℃ and 12,000×g for 20 min, and the supernatant was taken for the determination solution. A zinc acetate absorption well was placed in the small test tube to be installed, and then 100μL 30 mM $FeCl_3$ (dissolved in 1.2 MHCl) and 100μL 20 mM N, N-dimethyl14 - p-phenylenediamine (dissolved in 7.2 M HCl) were added to the test liquid. The sealing film of the test tube will react quickly at 37℃ for 30 min. At the wavelength of 670 nm, the absorbance was determined to find the corresponding H_2S concentration and calculate the content according to the prepared standard curve.

RNA extraction, library construction, and sequencing

RNA extraction from the 12 samples was performed by Biomarker Technologies Co., Ltd. (http://www.biomarker.com.cn/, Beijing, China). Total RNA purity and concentration were measured using a Nano-Drop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and RNA integrity was assessed using an Agilent 2100 Bioanalyzer/Lab Chip GX (Agilent, Santa Clara, CA, USA). After confirming total RNA quality, cDNA library construction and sequencing were performed. Sequencing was performed using the Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) in PE150 mode to obtain raw data. The raw data were then filtered to remove reads containing adapters and low-quality sequences (Include reads with a removal ratio of N greater than 10% and reads where the number

of bases with quality value Q≤10 constitutes more than 50% of the entire read.), resulting in high-quality clean reads. These clean reads were aligned to the reference genome of *Capsicum annuum* (*Capsicum_annuum* GCF_000710875.1 genome. fa) using HISAT2 for subsequent analysis [29]. Genes were identified as differentially expressed using thresholds of Fold Change≥1.5 and *Pvalue<0.01*. Differential expression analysis and functional annotation analysis of differentially expressed genes (DEGs) were performed based on DEG expression.

Validation of transcriptome data by qRT-PCR

To verify the reliability of the data, this study selected 7 antioxidant genes and H_2S synthesizing genes for qRT-PCR to verify the transcriptomic data according to the trend of antioxidant enzyme activity. RNA samples were provided by Biomarker Technologies Co., Ltd., and cDNA was synthesized from total RNA using a HiScript II Q Select RT Super Mix qPCR kit (Vazyme, Nanjing, China). Primers (Additional File 1, Supplementary Table 1) were designed by National Center for Biotechnology Information (NCBI: https://www.ncbi.nlm.nih.gov/). qRT–PCR was performed on the Light-Cycler 480II (Roche, Basel, Switzerland), using AceQqPCR SYBR Green Master Mix (Vazyme, China). The PCR reaction conditions were as follows: denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. To determine relative fold differences for each sample, the threshold constant (Ct) value was normalized to the Ct value of UBI-3, and set relative to control samples (0 h) according to the formula $2^{(-\Delta Ct)}$ [30]. Three independent technical replicates were analyzed for each sample.

Statistical analysis

All data were sorted using Excel 2016 software and analyzed using one-way analysis of variance (ANOVA) with SPSS 26.0 (IBM Corporation, Armonk, NY, USA). Duncan's new complex range method was applied at a 5% level of significance (*P<0.05*). Data are presented as the mean±Standard Error (SE, *n*=3). Graphs were created using Origin 21 and Adobe Illustrator.

Results

Effects of H_2S on membrane lipid peroxidation and ROS under cold stress

Under cold stress alone, REC initially increased then decreased over time, whereas MDA and Pro levels gradually increased over time. Relative to the Cold treatment, the H_2S treatment exhibited significantly lower REC, by 52.94%, 24.59%, and 16.36%, at 6, 24, and 48 h, respectively, however, there was no significant increase at 12 h (Fig. 1a), as well as significantly lower MDA content, by 31.54%, 60.63%, 44.50%, and 46.12%, at 6, 12, 24 and 48 h, respectively (Fig. 1b). Relative to the Cold treatment, the H_2S treatment exhibited significantly higher Pro content, by 19.07%, 68.46%, and 34.06%, at 12 h and 24 h (Fig. 1c). However, relative to the Control and Cold treatments, the HT group exhibited higher REC, MDA levels and Pro content.

ROS are key indicators that respond directly to cold stress, with $O_2^{\cdot-}$ and H_2O_2 being the predominant forms. Under cold stress, $O_2^{\cdot-}$ and H_2O_2 increased initially, then decreased. Relative to the Control and Cold treatments, the H_2S treatment exhibited significantly lower $O_2^{\cdot-}$ content, by 7.62% and 20.85%, at 48 h (Fig. 1e). Relative to the Cold treatment, the H_2S treatment achieved significantly lower H_2O_2 content at 24 h and 48 h, by 57.51% and 68.48%, respectively (Fig. 1d). However, under HT treatment, $O_2^{\cdot-}$ and H_2O_2 levels were significantly elevated, exacerbating the damage caused by ROS. These findings reveal that H_2S can alleviate cold stress by reducing REC and MDA levels, increasing Pro content to maintain osmotic stability, and reducing ROS accumulation.

Effects of H_2S on antioxidant enzyme activity in capsicum seedlings under cold stress

Under cold stress, SOD activity initially increased and then decreased, POD activity gradually increased, CAT activity gradually decreased, and APX activity initially increased and then decreased (Fig. 2a–d). There were no distinct trends in DHAR and MDHAR activity, although their activity was significantly higher under cold stress than in the normal control. H_2S treatment significantly increased the activity of the other enzymes. After 48 h of cold stress, relative to the Control, SOD was elevated in the HT and H_2S treatments, by 37.19% and 37.03%, respectively, and POD activity was elevated by 44.39% and 55.95%, respectively (Fig. 2a, b). CAT activity was lower under the H_2S treatment than in the control, but only at 12 h of cold stress, and was significantly higher than in the Control and Cold groups at the other time points (Fig. 2c). After 24 h of cold stress, APX and DHAR enzyme activity peaked in the H_2S group, exhibiting values that were 47.07% and 15.01% higher than in the Control and 27.21% and 26.50% higher than in the cold stress group, respectively (Fig. 2de). Although MDHAR activity was also elevated, the difference was not significant (Fig. 2f). In contrast, the HT group exhibited the opposite trend.

Effects of H_2S on the AsA–GSH system in capsicum seedlings under cold stress

Cold stress reduced the levels of AsA (a component of the AsA–GSH system), relative to the Control. H_2S treatment increased AsA content, but the increase was significant only after 6 h of cold stress. Under cold stress, H_2S treatment did not alter DHA content, whereas it increased the AsA/DHA ratio significantly, by 361.72%

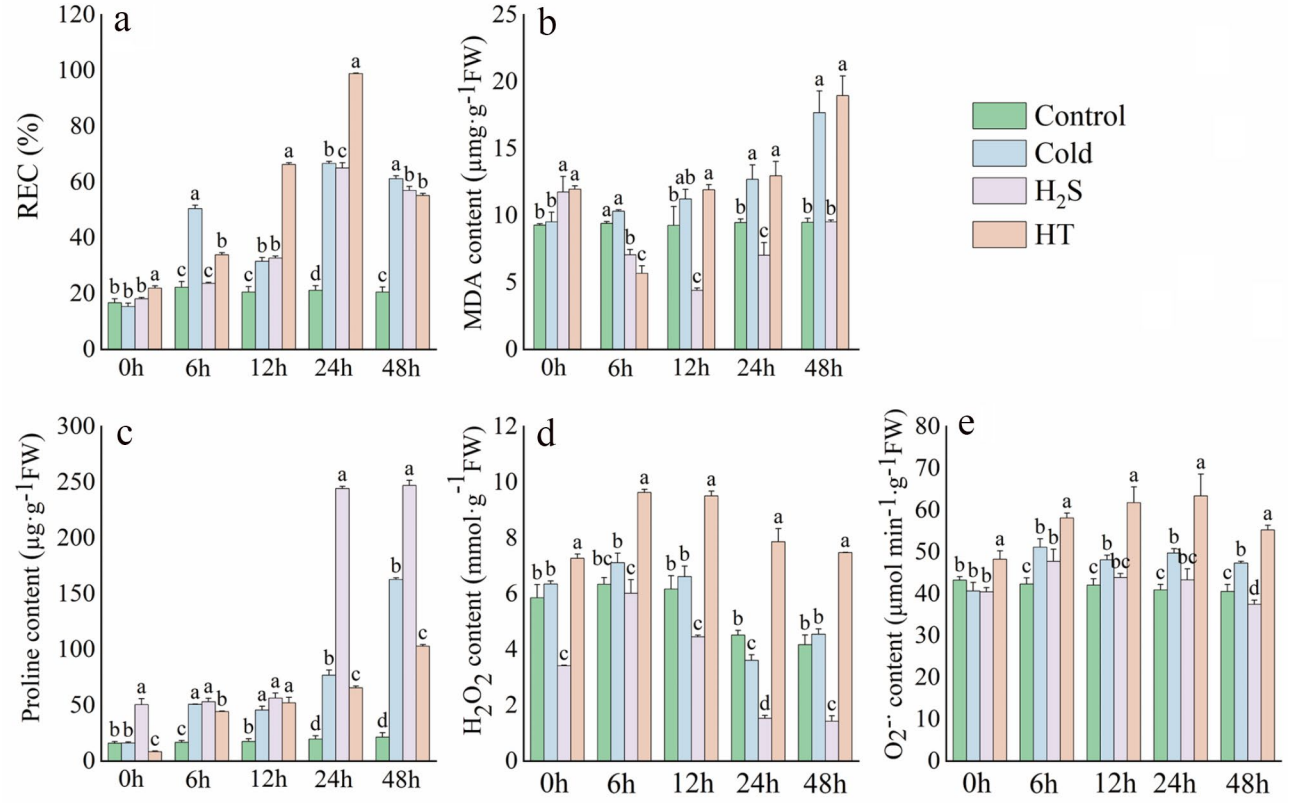

Fig. 1 Effects of H_2S on membrane lipids and ROS in capsicum plants under cold stress. **a**: Relative electrical conductivity (REC); **b**: Malondialdehyde (MDA); **c**: Proline content; **d**: H_2O_2; **e**: O_2^-

and 491.90%, at 6 and 12 h, respectively (Fig. 3a). Relative to the Control and Cold groups, the H_2S group exhibited significantly higher GSH and GSSG content: after 12 h of cold stress, GSH content was higher following H_2S treatment, by 610.50% and 126.43%, respectively, than in the Control and Cold groups; similarly, after 24 h of cold stress, GSSG content was higher following H_2S treatment, by 81.59% and 35.26%, respectively (Fig. 3b). Treatment with H_2S significantly increased the GSH/GSSG ratio under cold stress. This indicates that H_2S alleviates the effects of cold stress on capsicum by regulating AsA and GSH content, thereby altering the AsA/DHA and GSH/GSSG ratios.

Effects of H_2S on photosynthesis-related enzyme activity and photosynthetic parameters in capsicum seedlings under cold stress

Under cold-stress, H_2S improved photosynthetic parameters and Calvin-cycle enzyme activity. Under cold stress, the net photosynthetic rate (Pn), stomatal conductance (Gs), and transpiration rate (Tr) decreased significantly over time, whereas intercellular CO_2 concentration (Ci) increased (Fig. 4a–d). Relative to the Cold group, H_2S pretreatment achieved significantly higher Pn, Gs, and Tr, and lower Ci, following 6–48 h of cold stress. After 24 h of cold stress, the H_2S group exhibited higher Pn, Gs, and Tr, by 82.60% (Fig. 4a), 44.21%, and 55.37% (Fig. 4b and c), respectively, and a reduction in Ci of 21.47% (Fig. 4d). In contrast, the HT group exhibited the opposite trends in the gas-exchange parameters.

Under cold stress, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) activity initially declined then increased, while Fructose-1,6-diphosphate aldolase (FBAase) activity decreased continuously and Sedoheptulose1,7-diphosphate (SBPase) activity increased continuously (Fig. 4e–g). Relative to the Cold group, the H_2S group exhibited higher RuBisCO activity (by 13.34%; Fig. 4e), FBAase activity (by 12.35%; Fig. 4f), and SBPase activity (by 4.63%; Fig. 4g) after 24 h of stress. However, relative to the Control and Cold groups, HT treatment achieved significantly lower activity of all three enzymes. These results indicate that exogenous H_2S can mitigate the effects of cold stress on photosynthesis in capsicum seedlings.

Effects of H_2S on endogenous H_2S in capsicum seedlings under cold stress

Relative to the Control group, the Cold group exhibited significantly higher endogenous H_2S content after 0–12 h of stress, whereas the H_2S group exhibited elevated

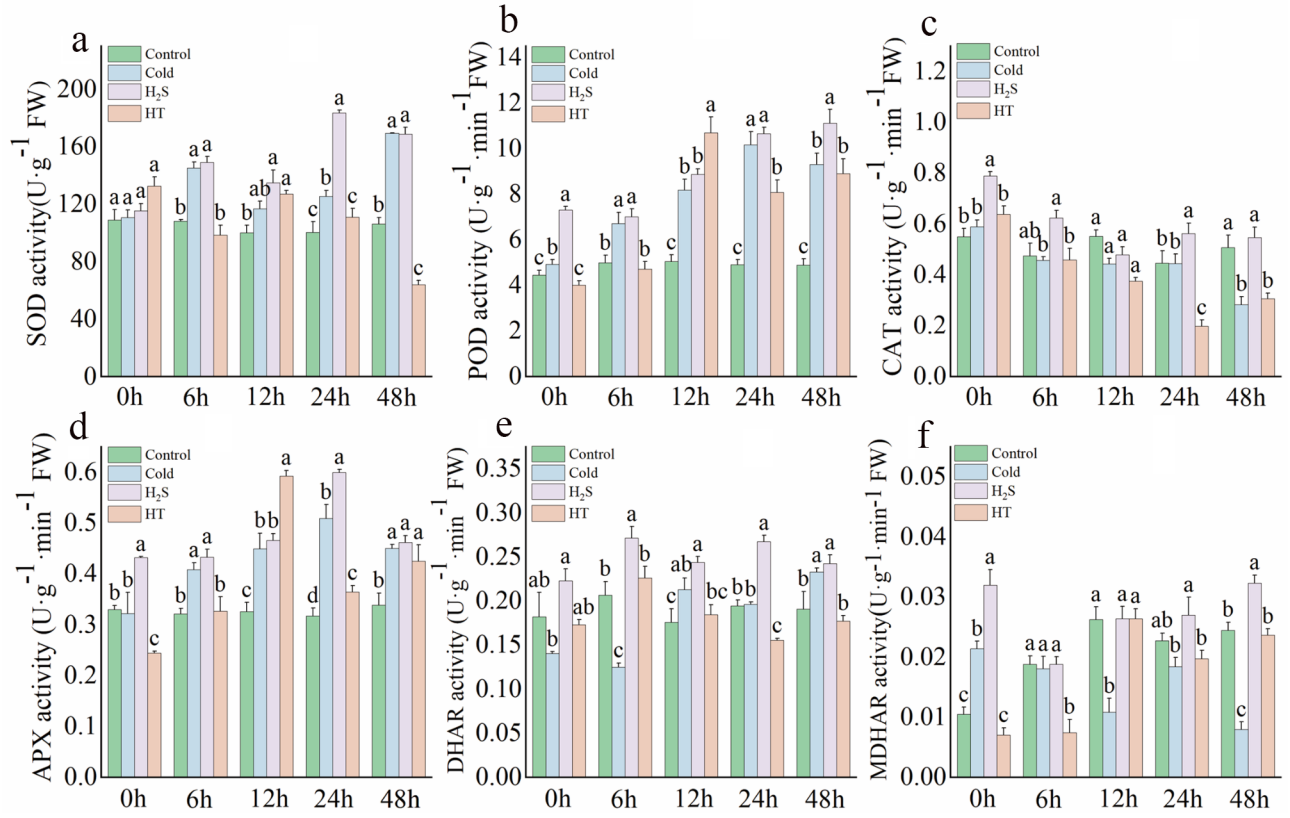

Fig. 2 Effects of H_2S on antioxidant enzyme activity in capsicum seedlings under cold stress. **a**: SOD; **b**: POD; **c**: CAT; **d**: APX; **e**: DHAR; **f**: MDHAR

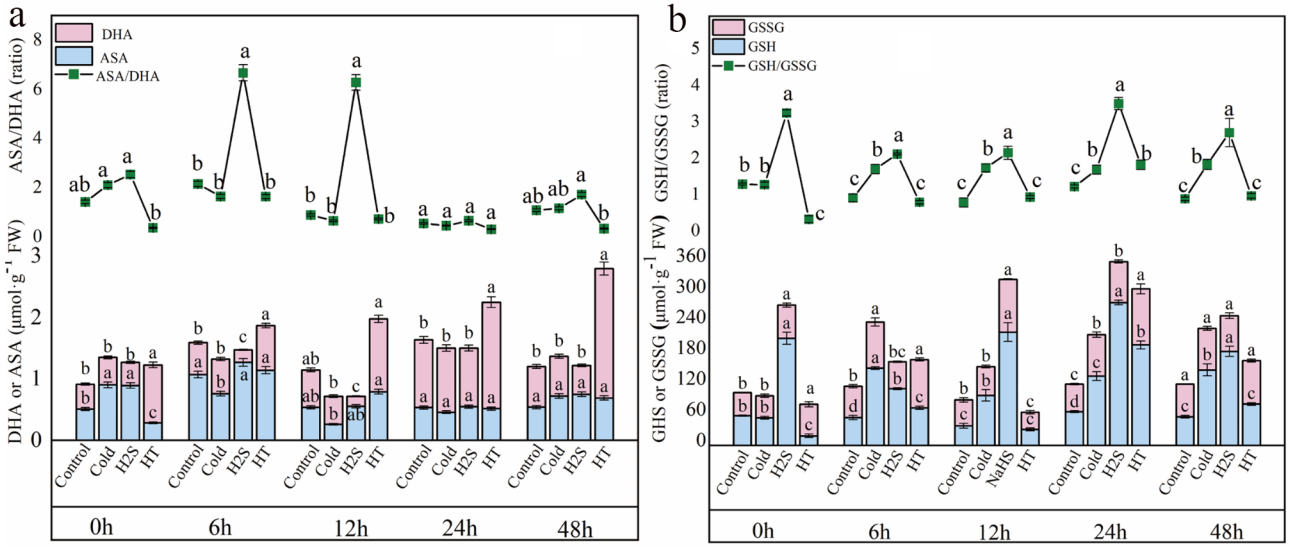

Fig. 3 Effects of H_2S on the AsA–GSH system in capsicum seedlings under cold stress. **a**: AsA and DHA content and AsA/DHA ratio; **b**: GSH and GSSG content and GSH/GSSG ratio

endogenous H_2S content at all time points. In contrast, relative to the Control group, HT treatment either reduced or maintained endogenous H_2S levels (Fig. 5a). LCD and DCD are key enzymes involved in the synthesis of endogenous H_2S in plants. Under cold stress, LCD and DCD activity first increased and then decreased in the in H_2S group, peaking at 24 h, with increases of 69.41% and 36.01%, respectively, relative to the Control group, and 71.39%, and 31.71%, respectively, relative to the Cold group (Fig. 5b, c). This indicates that exogenous H_2S not

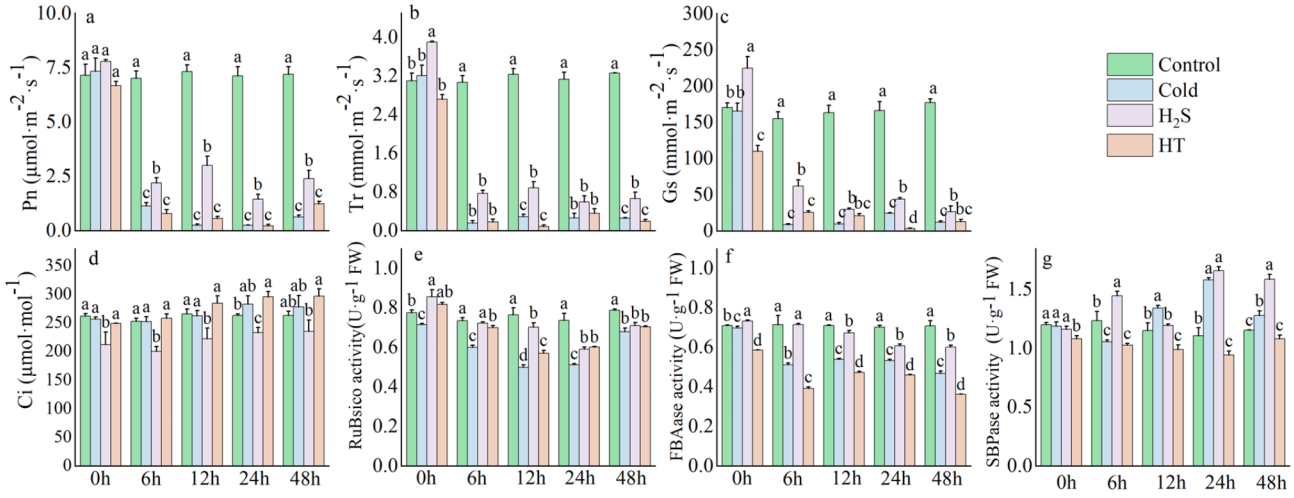

Fig. 4 Effects of cold stress on photosynthetic parameters and Calvin-cycle enzyme activity in capsicum seedlings. **a**: Net photosynthetic rate (Pn); **b**: Transpiration rate (Tr); **c**: Stomatal conductance (Gs); **d**: CO_2 concentration (Ci); **e**: RuBisCO activity; **f**: Fructose-1,6-diphosphate aldolase (FBAase) activity; **g**: Sedoheptulose1,7-diphosphate (SBPase) activity

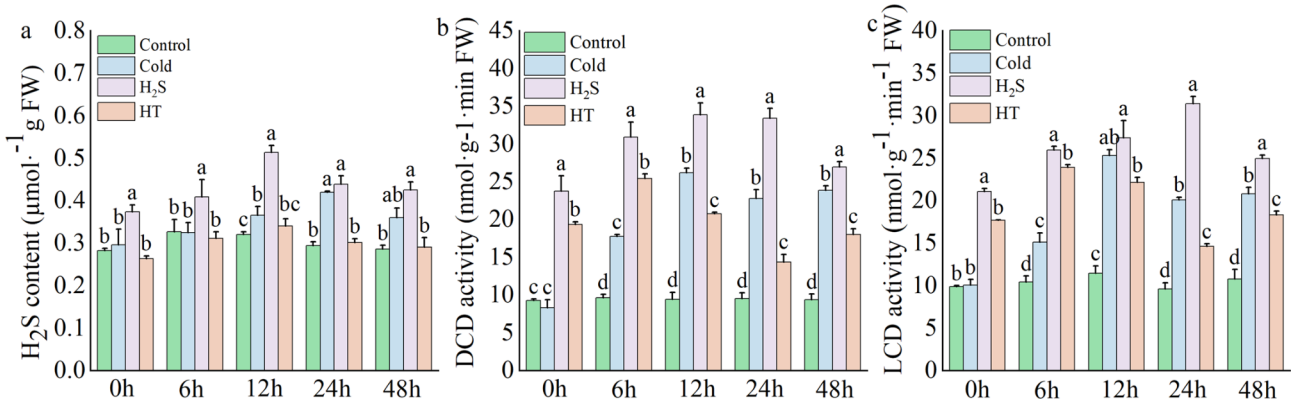

Fig. 5 Effects of H_2S on endogenous H_2S content in capsicum under cold stress. **a**: Endogenous H_2S content; **b**: LCD; **C**: DCD

only promotes the accumulation of endogenous H_2S during cold stress but also increases endogenous H_2S levels under normal conditions, while simultaneously enhancing the activity of synthesis enzymes (LCD and DCD).

Transcriptome library construction, quality assessment, and GO and KEGG analysis

RNA-seq analysis using the eukaryotic reference transcriptome for the 12 samples yielded 76.23 Gb of clean data, with each sample generating at least 5.81 Gb of clean data. At least 92.33% of the bases were Q30 bases. The clean reads from each sample were aligned to the designated reference genome, with alignment efficiencies of 90.84–95.36% (Additional File 1, Supplementary Table 2). Correlation analysis revealed that the correlation coefficient of the biological replicates, comparing the different treatments, reached 0.95 (Fig. 6a), demonstrating the strong reproducibility of the data. In the Principal Component Analysis (PCA), PC1 accounted for 33.47% and PC2 for 13.42% of the variance (Fig. 6b), indicating similarity in gene expression among the biological replicates. These results confirm the high reproducibility of the samples and highlight the variation in gene expression between the different samples.

Using the Gene Ontology (GO) database, GO enrichment analysis was conducted, considering the Biological Process (BP), Molecular Function (MF), and Cellular Component (CC) terms and comparing the Control and Cold treatments and the Cold and H_2S treatments (Fig. 6c, d). The most enriched BP categories were reproduction, immune system processes, and behavior. The most enriched CC categories were intracellular, protein-containing complex, and cellular anatomical entity, and the most enriched MF categories were catalytic activity, structural molecule activity, and transporter activity. We further mapped the DEGs to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to analyze the metabolic pathways. In the Control vs. Cold treatment comparison, 3707 DEGs were enriched in 134 metabolic pathways (Additional File 1, Supplementary Table 3),

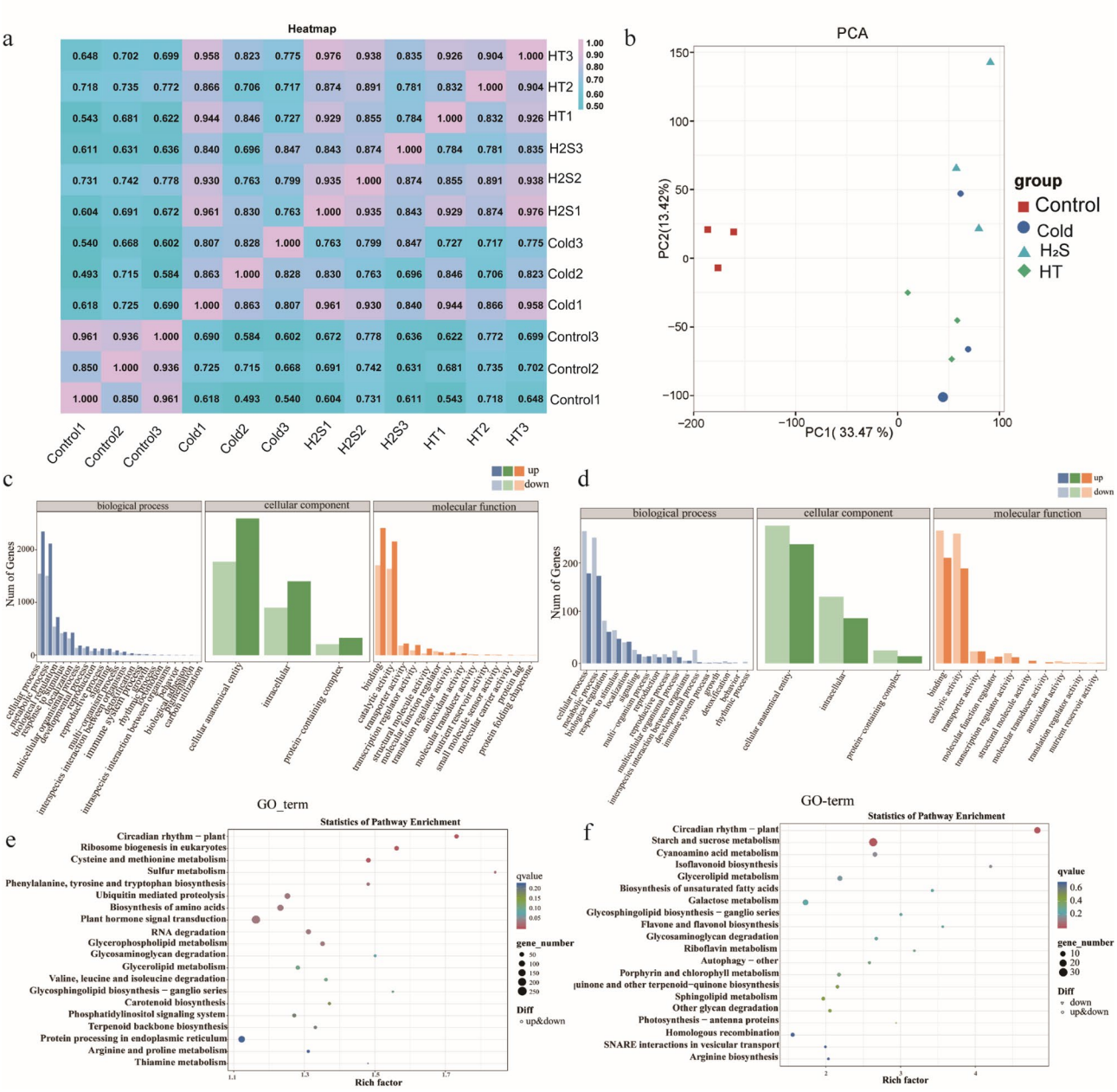

Fig. 6 Transcriptome sample correlation analysis, principal component analysis, and GO and KEGG enrichment analyses. **a**: Correlations between samples; **b**: Principal Component Analysis (PCA); **c**: GO enrichment analysis (Control vs. Cold treatments); **d**: GO enrichment analysis (Cold vs. H_2S treatments); **e**: KEGG enrichment analysis (Control vs. Cold treatments); **f**: KEGG enrichment analysis (Cold vs. H_2S treatments)

with the top five most significantly enriched pathways being plant-circadian rhythm, ribosome biogenesis in eukaryotes, cysteine and methionine metabolism, sulfur metabolism, and phenylalanine, tyrosine, and tryptophan biosynthesis. In the Cold vs. H_2S treatment comparison, 416 DEGs were enriched in 105 metabolic pathways (Additional File 1, Supplementary Table 4), with the top five most significantly enriched pathways being plant circadian rhythm, starch and sucrose metabolism, cyano-amino acid metabolism, isoflavonoid biosynthesis, and glycerolipid metabolism (Fig. 6e, f).

Effects of H_2S on starch sucrose metabolism and the ascorbate–glutathione pathway in capsicum seedlings under cold stress

Transcriptome data analysis of cold-sensitive capsicum seedlings treated with H_2S under cold stress identified 33 DEGs related to the cold stress response and sugar metabolism. Seven of these DEGs encode sucrose

synthase (*SUS*), which promotes the synthesis of UDP-glucose (five upregulated and two downregulated). Following H_2S treatment, ectonucleotide pyrophosphatase, which promotes the synthesis of the starch and cellulose glycosyl donors ADP-glucose and GDP-glucose, was upregulated; two genes encoding beta-fructofuranosidase (*INV*), which promotes the formation of D-fructose and D-glucose, were upregulated; one alpha-glucosidase gene, which prevents the hydrolysis of glucosidic bonds downregulation of three endoglucanase genes, was downregulated; and seven beta-glucosidase (*bglX*) genes were differentially regulated (one upregulated and six downregulated). Following H_2S treatment, five differentially regulated glucan endo-1,3-beta-glucosidase 1/2/3 (*GN1_2_3*) genes were identified (two upregulated and three downregulated); these inhibit cellulose decomposition and affect sucrose breakdown into D-glucose (Fig. 7, a); and trehalose 6-phosphate synthase (*TPS*) was downregulated. Although by NaHS treatment promoted ADP-glucose formation, it affected the efficiency of the conversion of photosynthetically produced sucrose into starch by downregulating starch synthase (*glgA*) and glycogen phosphorylase (*PYG*) genes, while upregulating the alpha-amylase (*AMY*) gene, thus accelerating the conversion of starch into maltose. H_2S induced downregulation of the alpha-glucosidase (*malZ*) gene, thus hindering the breakdown of maltose into D-glucose.

Metabolic pathway analysis following H_2S treatment identified 18 common DEGs related to GSH and ascorbic acid (Fig. 7b). Among these, two DEGs encoding gamma-glutamyl-transpeptidase 3 (*GGT*) were downregulated, whereas eight encoding glutathione S-transferase (*GST*) were upregulated, promoting the synthesis of R-S-glutathione; and two DEGs encoding L-ascorbate peroxidase (*APX*), regulating the conversion of H_2O_2 to H_2O and the formation of DAH from AsA, were identified, with one upregulated and one downregulated. One of the identified DEGs, encoding glucose-6-phosphate 1-dehydrogenase (*G6PD*), participates in promoting the conversion of GSSG back to GSH.

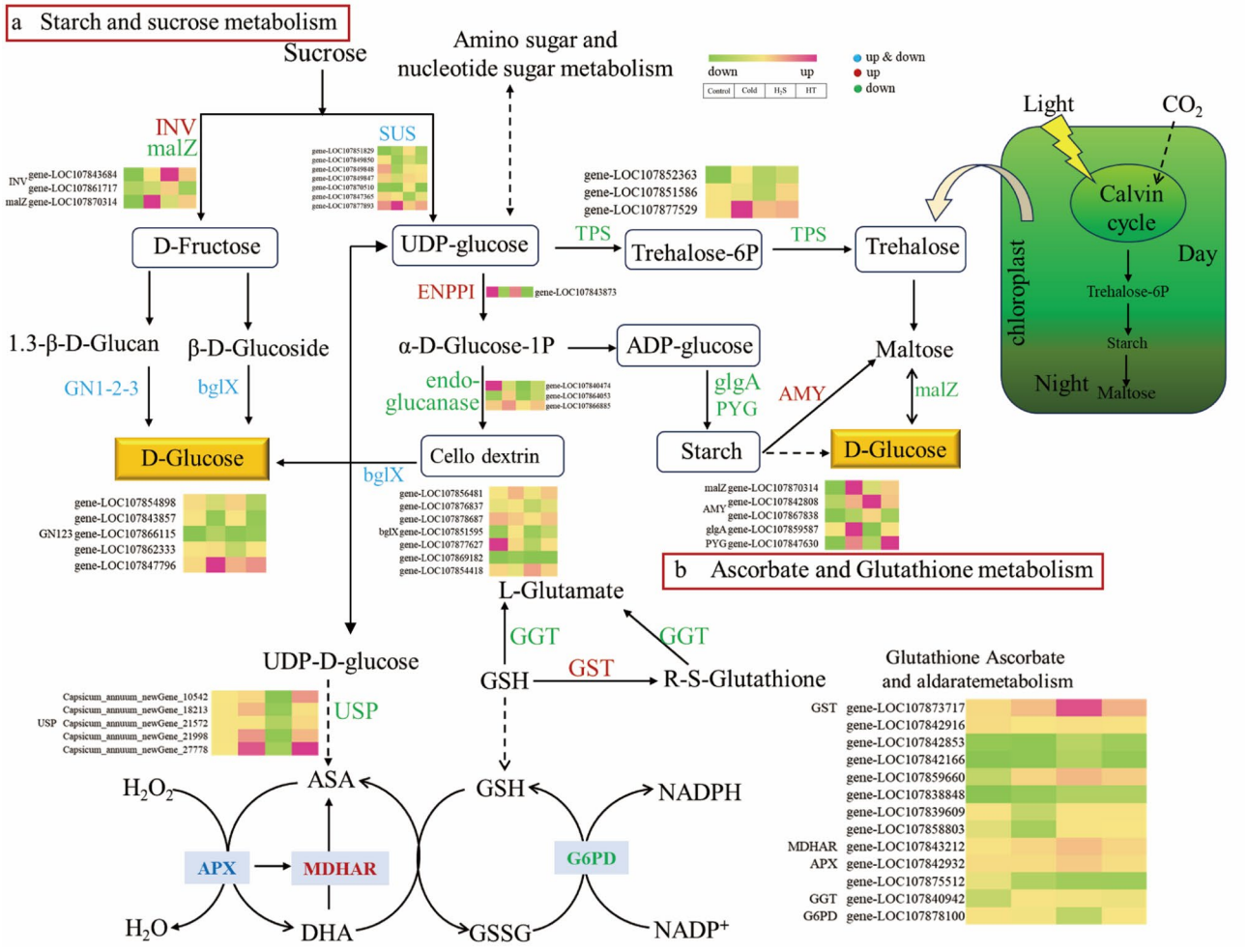

Fig. 7 Analysis of differentially expressed genes (DEGs) involved in the metabolism of starch, sucrose, ascorbate, and glutathione. **a**: Starch and sucrose metabolism; **b**: Ascorbate and glutathione metabolism. Blue: Upregulated or downregulated; Red: Upregulated; Green: Downregulated

Effects of H_2S on plant hormone signal transduction in capsicum seedlings cold stress

Transcriptome analysis of capsicum seedlings treated with H_2S identified DEGs related to hormone signal transduction in response to cold stress, mainly involving stimulators (such as Aux, GA, cytokinin [CTK], and MEL) and inhibitors (such as ETH, ABA, SA, and JA). Following H_2S treatment, in the Aux signal-transduction pathway, one DEG encoding a growth protein (*Aux/IAA*) with inhibitory function and one DEG related to the *SAUR* family protein were upregulated; one gene encoding the TIR1 receptor protein was downregulated; and two genes encoding *ARF* family proteins were downregulated. In terms of CTK synthesis, two genes encoding histidine-containing phosphotransfer proteins (*AHP*) were upregulated by H_2S treatment, and four genes in the two-component response regulator *ARR-B* family were differentially regulated (one upregulated and three downregulated), collectively modulating CTK under cold stress. In terms of GA synthesis, two DEGs encoding *DELLA* proteins were downregulated by H_2S treatment. In terns of ABA synthesis, H_2S treatment upregulated one DEG (*PYR/PYL*); when phosphorylated by ABA-receptor kinases, *PYR/PYL* enhances *PP2C* inhibition and leads to the activation of the ABA signal. BR binds to the extracellular domain of *BRI1* and activates its intracellular domain. Activated *BRI1* then phosphorylates its negative regulator, *BKI1*, allowing it to bind to its coreceptor *BAK1*. *BRI1* and *BAK1* phosphorylate each other to fully activate BR signaling. Following NaHS treatment, two DEGs encoding *BRI1* were upregulated and one downregulated, while four encoding *BAK1* were upregulated and two downregulated. Two DEGs encoding *bHLH* zip transcription factor (*MYC2*) regulate two branches of the JA signaling pathway. *MYC2* (*gene-LOC107850747*), which is negatively self-regulated, is essential for the expression of pathogen defense genes, whereas *EGL1* (*gene-LOC107865400*), which is positively regulated by *MYC2*, participates in the response of plants to cold stress. SA is an essential hormone related to disease resistance; within the transcription factor TGA family, *TGA7* (upregulated following H_2S treatment) and *TGA1* (downregulated) interact to activate PR gene expression.

Quantitative real-time PCR (q-PCR) validation

To verify the reliability of the data, this study selected 9 antioxidant genes and H_2S synthesizing genes for qRT-PCR according to the trend of antioxidant enzyme activity. Relative to the Control and Cold groups, the H_2S group exhibited significantly higher FPKM values and RNA-seq-based expression of *LCD*, *CDD1*, *DCD2*, *APX*, *MDHAR*, *GR*, *GPX*, *GSH*, and *GST* (Fig. 8), and these levels exhibited the same trends, indicating that the samples generated reliable RNA-Seq data.

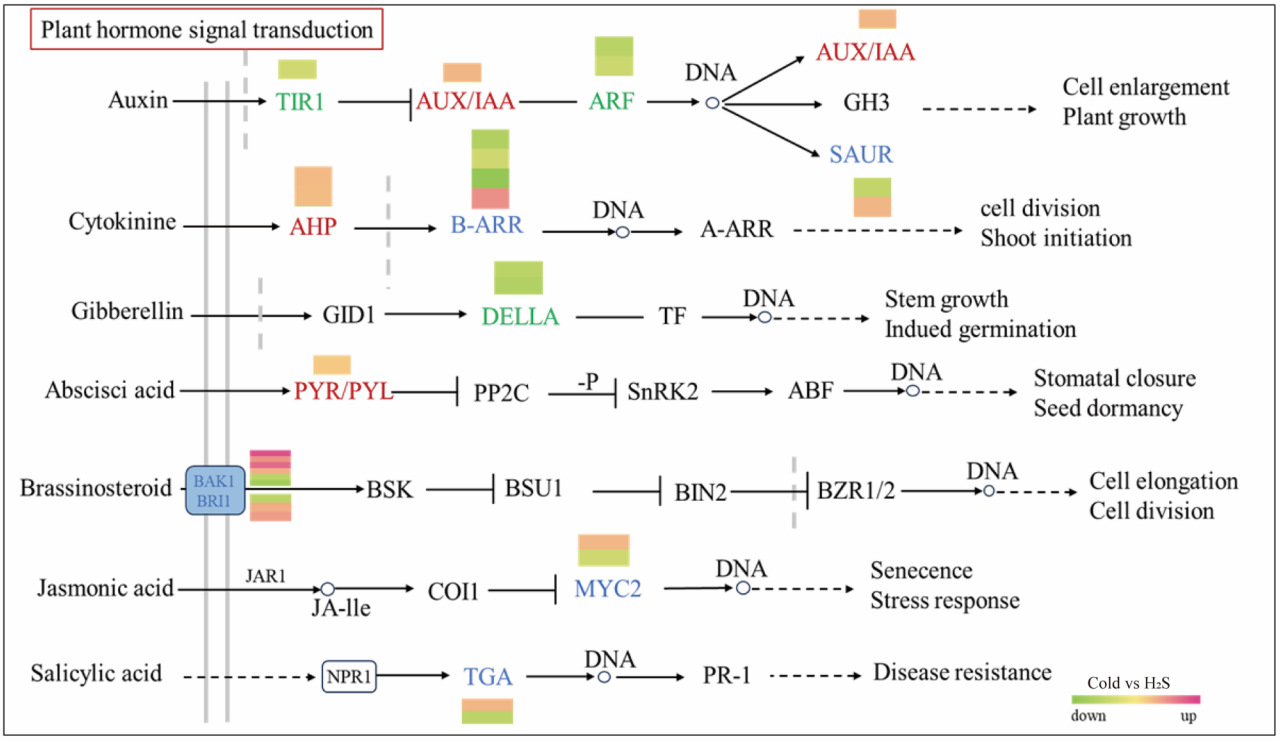

Fig. 8 Quantitative real-time PCR (q-PCR) validation

Discussion

Capsicum, a warm-loving vegetable crop, is highly sensitive to temperature. Cold stress can have different detrimental effects on capsicum plants depending on the growth stage. Cold damages membrane lipids, increasing membrane permeability and leading to cellular metabolic disorders. REC and MDA are commonly used indicators of membrane lipid damage, whereas Pro acts as an osmoprotectant [31]. In studies on H_2S alleviating aluminum stress in rice, it was found that H_2S can enhance root elongation, reduce aluminum content in root tips, decrease MDA and H_2O_2 levels, and increase the activity of antioxidant enzymes [32]. Here, as shown in Fig. 1, H_2S application reduced REC and MDA levels following cold stress. Additionally, it increased Pro content, which is consistent with the results observed in cucumbers under low-temperature stress [33]. This suggests that H_2S helps to maintain membrane lipid integrity in capsicum seedlings and increases the active osmotic capacity of Pro, thereby enhancing its tolerance to cold injury. When ROS accumulation leads to oxidative stress, the elevated H_2S levels can be reduced via both enzymatic and non-enzymatic pathways [34]. exogenous H_2S can reduce the accumulation of H_2O_2 and $O_2^{\cdot-}$ in barley roots [35] and also enhance antioxidant enzyme activity in wheat [36]. Here, in capsicum seedlings, cold injury resulted in excessive production of ROS-related $O_2^{\cdot-}$ and H_2O_2. H_2S application alleviated these effects by activating antioxidant enzymes (namely SOD, POD, CAT, APX, DHAR, and MDHAR) to continuously reduce or eliminate ROS levels and to maintain ROS homeostasis physiologically stable levels. SOD primarily converts $O_2^{\cdot-}$ into H_2O_2, which is then reduced to H_2O by electrons provided by APX (as shown in Fig. 2). Studies on aluminum stress in soybeans and cadmium stress in Bermuda grass have generated similar conclusions on the role of H_2S [35, 37].

The non-enzymatic antioxidant system, also known as the AsA–GSH cycle, is one of the main systems of plant defense against stress. GSH is a small redox-active molecule that occurs primarily in two stable forms, GSH and GSSG. DHA is a reversibly oxidized form of AsA. GSH participates in the AsA–GSH cycle [38]. DHA can maintain α-tocopherol and zeaxanthin in their reduced states and protect proteins from denaturation via oxidation of thiol groups, thereby preserving cell membrane integrity [35]. Here, H_2S regulated the non-enzymatic antioxidants AsA, DHA, GSSG, and GSH in the AsA–GSH cycle in capsicum seedlings (as shown in Fig. 3). Similar conclusions were drawn by Kaya et al. in their study on capsicum under high zinc stress [39]. H_2S treatment upregulated *GST*, *MDHAR*, and *APX* and downregulated *GGT*, *USP*, and *G6PD* (as shown in Fig. 7b), promoting the conversion of H_2O_2 to H_2O. This indicates that both enzymatic and non-enzymatic systems are involved in protecting the membrane integrity of pepper seedlings. In rice leaves, H_2S improves photosynthetic capacity by increasing the stomatal aperture and density. In *Arabidopsis*, in contrast, NaHS inhibits NO production and induces stomatal closure [40], and H_2S reduces stomatal diameter and alleviates cold damage [41]. The role of H_2S in stomatal dynamics therefore remains controversial. Here, cold treatment rapidly reduced Pn, Gs, Tr, and Ci levels, whereas H_2S application significantly increased Pn, Gs, and Tr levels, indicating that H_2S can promote stomatal opening and enhance the photosynthetic capacity of capsicum seedlings under cold stress. Non-stomatal factors are the primary factors causing the long-term reduction in photosynthetic capacity at low temperatures [42]. H_2S can promote CO_2 transport, upregulate photosynthesis-related enzymes, and mediate thiol group redox reactions to improve photosynthesis [43]. Following cold stress, RuBisCO, FBA, and SBP activity was reduced, while H_2S increased this activity, promoting CO_2 fixation via the dark reaction. In maize leaves under iron deficiency, application of H_2S upregulated the protein and gene expression of RuBisCO and phenylpyruvate decarboxylase [44].

Starch plays a significant role in regulating stress tolerance, by releasing energy, sugars, and derivative metabolites via its degradation [45]. When sucrose enters the vacuole, it is converted into glucose and fructose by invertase (*INV*) and sucrose synthase (*SUS*) [11]. In moss, ABA-induced starch degradation improves cold tolerance [46], and drought-resistant beans can degrade more starch than drought-sensitive beans [21]. The activity of *AMY*, an endohydrolase that cleaves α-1,4-glycosidic linkages in starch, is associated with starch degradation [47]. Here, H_2S upregulated *INV* and *SuS* in the sucrose-metabolism pathway, thus regulating endoglucanase enzyme-encoding genes such as *GN123*, *bglX*, *ENPPI*, and genes encoding cellulose synthesis-related endoglucanase, as shown in Fig. 7, thus promoting monosaccharide accumulation in capsicum seedlings. H_2S downregulated genes encoding starch-related enzymes, including *glgA* and *PYG*, the seaweed sugar-related gene *TPS*, and *malZ*, but upregulated *AMY*. This indicates that, in capsicum seedlings, H_2S primarily regulates starch breakdown by regulating *AMY* to facilitate the conversion of starch into sugar, thereby enhancing osmotic stability and cold tolerance. This indicates that the use of H_2S can regulate the breakdown of accumulated starch and sucrose in pepper seedlings into monosaccharides for absorption, thereby maintaining the energy supply of pepper seedlings after cold stress and enhancing their cold tolerance.

Crosstalk between H_2S and hormones comprises one of the most important signaling mechanisms in plants [48, 49]. Plant hormones include stimulators such as Aux, GA, CTK, and MEL, and inhibitors such as ETH, ABA, SA,

and JA (Fig. 9) [50]. Here, NaHS activated downstream stimulators, including the auxin-responsive protein *IAA* and *SAUR* family proteins, promoting *Aux* synthesis; it upregulated CTK synthesis-related *AHP* and *ARR-B* and enhanced their DNA transcription. NaHS downregulated *DELLA* proteins, in the GA synthesis pathway. In the presence of inhibitors, NaHS increases the inhibition of *PP2Cs* by activating ABA signaling (by upregulating *PYR/PYL* expression). For *Arabidopsis*, Aroca et al. reached a similar conclusion [51], finding that H_2S regulates guard-cell stomatal closure in response to cold stress via ABA signaling [20]. H_2S affects JA and SA synthesis by upregulating *MYC2* and *TGA*. Treating tomato seedlings with NAA upregulated *DES1* expression and DES activity, leading to the accumulation of endogenous H_2S, which stimulates lateral-root growth [52]. In tomatoes, H_2S and GA mutually promoted each other to effectively alleviate boron stress in tomatoes, whereas HT eliminated this effect [53]. Aroca et al. [51] observed reduced persulfidation of the ABA receptors *PYR1*and *PYL1* in *Arabidopsis DES1*-knockout mutants. In the epidermis of the faba bean, *Arabidopsis*, and *Impatiens*, H_2S promotes stomatal closure by regulating the *ABA*-dependent pathway and *ABC* transporters located in guard cells [46]. In maize seedlings under heat stress, SA activates endogenous H_2S and its synthesizing-enzyme LCD, whereas H_2S does not

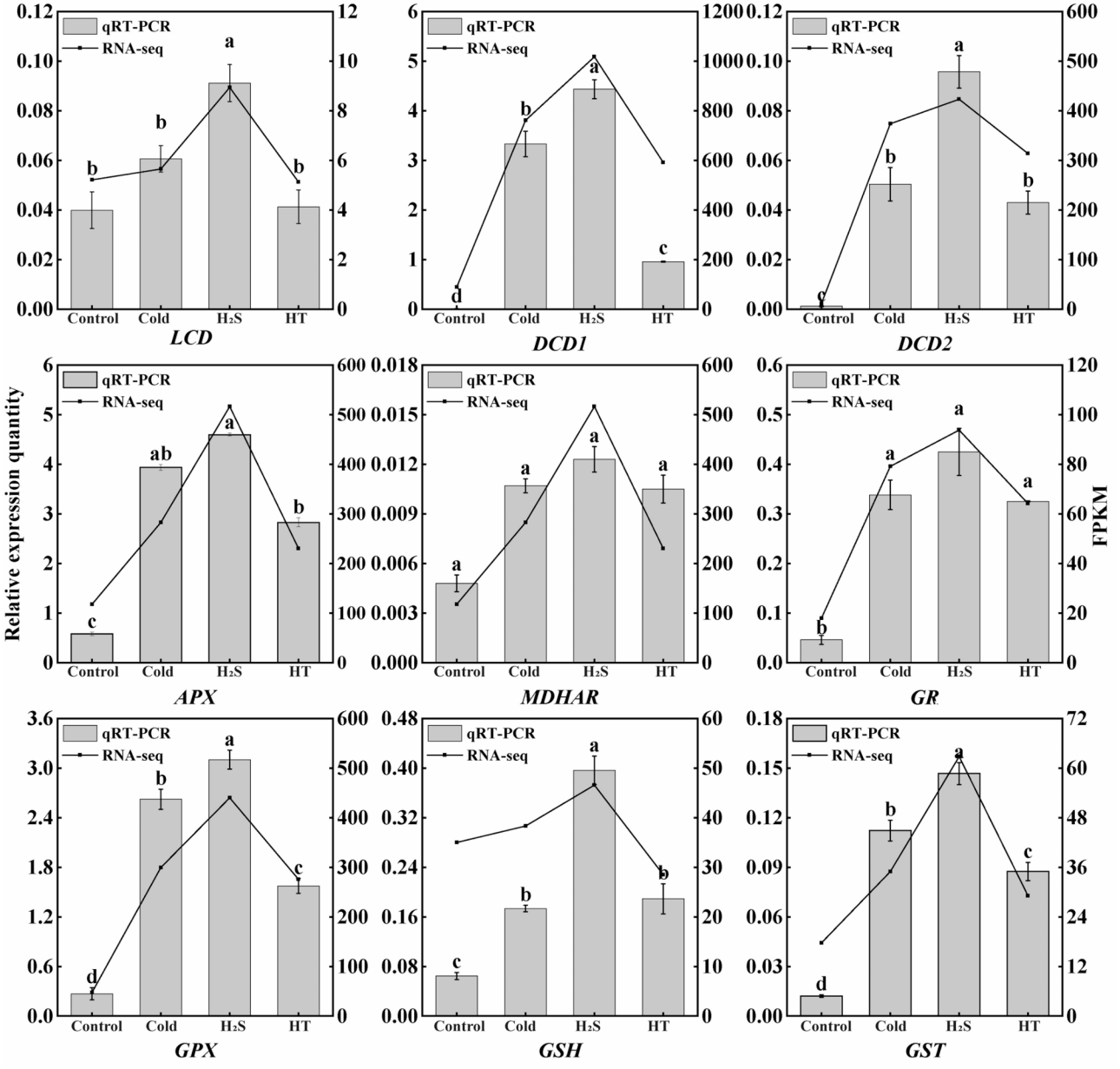

Fig. 9 Effects of H_2S on plant hormone signal transduction in capsicum seedlings under cold stress

significantly affect *PAL* and *BA2H* (the key enzymes in SA biosynthesis) or endogenous SA levels; H_2S thus acts as a downstream signal of SA to regulate plant physiological functions [54]. Therefore, in capsicum seedlings, H_2S alleviates cold stress by activating the ABA-dependent signaling pathway and promoting endogenous H_2S accumulation. H_2S pretreatment upregulates downstream transcription-factors expression under cold stress by promoting interactions between BR and the extracellular domain of *BRI1* and its co-receptor, *BAK1* [55]. *BRI1* and *BAK1* participate in the pathway regulating sugar responses via G protein subunits [56]. After being subjected to low-temperature stress, pepper seedlings exhibit changes in the levels of various plant hormones as part of their stress response mechanisms. Exogenous H_2S induces structural genes such as IAA and CTK, which are involved in regulating defense functions, thereby enhancing the cold tolerance of pepper seedlings.

Here, H_2S was applied to alleviate the effects of cold damage in capsicum seedlings. Based on these findings, H_2S regulates various plant hormones and carbon compounds such as starch and sucrose. This work provides some reference for using biotechnological methods to help vegetables or other plants resist cold stress. Future research will focus on in-depth studies of H_2S signaling molecules in balancing ROS mechanisms.

Conclusions

These findings elucidate the mechanisms whereby H_2S pretreatment regulates the alleviation of cold stress in cold-sensitive capsicum seedlings. The findings indicate that H_2S can alleviate damage to capsicum seedlings caused by cold stress by regulating the accumulation of physiologically active substances, maintaining membrane integrity, enhancing enzymatic and non-enzymatic antioxidant activity, maintaining ROS homeostasis, and improving photosynthesis. In capsicum seedlings under cold stress, H_2S transcriptionally upregulates key genes involved in sucrose and starch metabolism, ascorbic acid and GSH metabolism, and hormone-signaling pathways (including *GST*, *APX*, *MDHAR*, and *AMY*), while downregulating genes such as *GGT*, *G6PD*, *USP*, *glgA*, *PYG*, and *malZ*. H_2S thereby maintains sugar accumulation and osmotic stability, thus enhancing the cold tolerance of cold-sensitive capsicum seedlings.

Supplementary Information

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Additional file 1

Additional file 2

Additional file 3

Additional file 4

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Not applicable.

Author contributions
A: Conceptualization, X S. and B C.; B: methodology, Z X., L L. and J X.; C : software, W T., and M X.; D: validation, D W. and B C.; E: formal analysis, L Z. and Z H.; F: investigation, Y L. and B S.; G: resources, X S. and Z X.; H: data curation, X S., Z X and L Z.; I: writing—original draft preparation, X S.; J: writing—review and editing, X S. and B C.; K: visualization, B C., Z X. and Y T.; L: supervision, Y L., B S. and H L.; M: project administration, H L.; N: funding acquisition, H L.;

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

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